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[ ] PURIFICATION OF THIOLS FROM BIOLOGICAL SAMPLES

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The dominant low molecular weight thiol in most eucaryotes is glutathione but other thiols, including ones with unknown identity, occur in some eucaryotes and many procaryotes[1]. In order to identify novel thiols it is usually necessary to obtain a pure sample. In this chapter we describe a two step purification procedure which allows a low molecular weight thiol component in a biological extract to be isolated as the monobromobimane derivative (see this volume [XX] and [XX]) in highly purified form. The thiols present in a deproteinized extract were first isolated on a thiolagarose gel via a thiol-disulfide exchange reaction. The thiols were then eluted with dithiothreitol (DTT) and derivatized with monobromobimane (mBBr). The derivative was purified to homogeneity by preparative high-performance liquid chromatography (HPLC). A procedure for electrolytic reduction of the bimane derivative was developed which allows regeneration of the thiol form of the purified product. Application of the method is illustrated for isolation of a major thiol component found in Halobacterium halobium whose structure was shown to correspond to γ-glutamylcysteine[2].

Materials

Reagents and sources were: glacial acetic acid, pyridine, mercury, and ammonium hydroxide - Mallincrodt; diethylenetriaminepentaacetic acid (DTPA), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Trizma base (Tris), and 2,2'-dithiodipyridine - Sigma; high purity dithiothreitol (DTT) and monobromobimane (mBBr) - Calbiochem-Behring; acetonitrile and methanol - Burdick and Jackson; ethyl acetate - Pierce; Puriss grade methanesulfonic acid - Fluka. The 2-thiopyridyl mixed disulfide of thiopropyl-Sepharose 6B was obtained from Sigma or prepared from Sepharose 6B by the method of Axén, Drevin and Carlsson[3].

Purification

Halobacterium halobium R-1 cells (0.6 g wet weight) were dispersed with a Tekmar homogenizer into 5 ml of 50% aqueous acetonitrile containing 5 mM DTPA and 25 mM methanesulfonic acid while heating at 60°C for 10 min. After cooling on ice the sample was centrifuged 10 min at 14,000 x g in a Sorval RC-5 centrifuge. The clear supernatant contained 0.9 µmoles of thiol by DTNB titration[4] and 0.77 µmoles of

an unidentified thiol (later shown to be γ-glutamylcysteine) by derivatization with mBBr and HPLC analysis (peak eluting at 10 min, Fig. 1A). The supernatant was diluted two-fold into 200 mM Tris, pH 8.0, containing 5 mM DTPA and 1.0 mM DTT (added to reduce disulfides and protect thiols toward oxidation).

A 20 x 6 mm column was packed with 0.75 ml of 2-thiopyridyl activated thiopropyl-Sepharose 6B (25 µmoles disulfide) and equilibrated with 25% acetonitrile in water containing 100 mM Tris, pH 8.0, and 5 mM DTPA. The thiol containing supernatant was applied to the column at a flow rate of 0.5 ml per min after which the column was washed with 25 volumes of the equilibration buffer. Binding of thiol to the column was monitored based upon the absorbance of 2-mercaptopyridine ($\epsilon_{343} \cong 7000$)[5] in the effluent which indicated that ~100% of the applied thiol had reacted with the gel. The column was further washed with 25 column volumes of 50 mM Tris, pH 8.0, containing 1 mM DTPA and then eluted with 3mM DTT in the same buffer. The thiol content of the eluate was determined by titration with DTNB and 1.5 equivalents of mBBr added as a 200 mM solution in acetonitrile. Reaction was allowed to proceed 10 min in the dark and was stopped by addition of acetic acid to 1%. Excess mBBr and the mBBr derivatives of 2-thiopyridine and DTT were removed by extracting six times with an equal volume of ethyl acetate. The recovery of the unknown derivative as determined

by HPLC analysis using method 1 (see [XX] this volume) was 96% at this stage. The product was protected from light and lyophilyzed.

Preparative HPLC purification was accomplished with an Altex Ultrasphere ODS 5 μ 10 x 250 mm column operated at room temperature and a flow rate of 3 ml per min. Buffer components, selected for their volatility, were: buffer A - 0.25% aqueous acetic acid titrated to pH 3.5 with triply distilled pyridine; buffer B - methanol. The elution program with linear gradients was: 0 min, 10% B; 20 min, 25% B; 25 min, 100% B; 27 min 100% B; 28 min, 10% B; 40 min, reinjection. The derivatized and lyophilyzed thiopropyl-Sepharose eluate (see above) was taken up in 0.5% aqueous acetic acid, 100-200 μL aliquots were injected on the preparative column, and the peak eluting at 20 min was manually collected, pooled, and lyophilyzed. HPLC analysis of the purified product (Fig. 1B) showed that it was highly purified relative to the crude material and was obtained in an overall yield of >90%.

Electrolytic Reduction

A mercury pool electrolytic reduction cell similar to that used by Kadin and Poet[6] was constructed from a 30 mm section of 10 mm glass tubing fitted at one end with a rubber septum cap through which a platinum wire serving as cathode was inserted. Approximately 400 μl of mercury and a 1 x 3 mm magnetic stirring bar were placed in the tube and 1 ml of the thiol-mBBr derivative in 100 mM aqueous acetic acid added on top. A U-shaped 1/8 in. O.D. Teflon tube filled with 2% agar in saturated sodium chloride served as an agar bridge to connect the electrolysis cell with a 10 ml beaker containing saturated sodium chloride and the platinum wire anode. The electrodes were connected to a Hewlett-Packard Model 6299A power supply and the electrolysis performed at constant voltage. Voltages given below refer to applied voltage.

The progress of the reduction was monitored by HPLC analysis of the

remaining thiol-mBBr derivative and the thiol production was determined from the increase in the derivative found upon analysis after derivatization of the thiol formed with mBBr. At low voltage (~2 volts) reduction of an 0.2 mM solution of the GSH-mBBr derivative was slow ($t_{1/2}$ ~25 min) and HPLC analysis showed that formation of syn-(methyl,methyl)bimane occurred in consort with disappearance of the mBBr derivative and appearance of the free thiol. The yield of thiol was >95% yield when tested with the derivative of glutathione (see ref. 7 for a discussion of the electrochemistry of bimanes). At higher voltages (3-10 volts) the reaction was faster but the syn-(methyl,methyl)bimane itself was reduced to nonfluorescent products (Fig. 2), this process becoming so rapid at 10 volts that no syn-(methyl,methyl)bimane was detected during monitoring of the reaction progress by HPLC analysis. The yield also decreased with increasing voltage, the recovery of GSH from the corresponding derivative being 90% after 15 min at 3 volts and 75% after 10 min at 10 volts. Electrolytic reduction of the purified thiol derivative from H. halobium at 10 volts for 10 min resulted in a 75% recovery of the thiol. After lyophilization the product was oxidized to the corresponding disulfide in 1 ml of 0.1 M ammonium hydroxide under a stream of dry oxygen, the progress being monitored by titration with DTNB and the ammonium hydroxide being replenished as needed. This material was used for amino acid analysis, N-terminal amino acid determination, and other structural studies without removal of bimane coproducts and was shown to correspond to γ-glutamylcysteine[2]. Removal of syn-(methyl,methyl)bimane following reduction is possible by extraction with ethyl acetate but its electrolytic reduction products have not been identified and their removal may require an additional preparative HPLC step or other isolation procedure.

General Comments

The monobromobimane derivative of a thiol has unique characteristics which

make it especially attractive as the form to be purified in the isolation of an unknown thiol compound. Blocking of the thiol with the bimane group stabilizes it toward oxidation and provides a fluorescent label that can be easily followed and quantitated. The bimane label also greatly increases the affinity of simple ionic thiols for reversed phase HPLC column packings and enhances the degree of purification that can be obtained by preparative HPLC procedures. The fluorescent label allows the purified derivative to be readily characterized with respect to its behavior on electrophoresis and thin layer chromatography, and facilitates structural studies of the derivative. Finally, the ability to electrolytically remove the bimane label to regenerate the thiol provides access to the unmodified form of the unknown compound.

Monobromobimane labeling has proven useful in the labeling of protein thiol groups and in the study of the peptides derived from the labeled protein[8]. The present methods should extend this utility by providing a technique for the purification of cysteine-containing peptides generated in protein structural studies.

Acknowledgments

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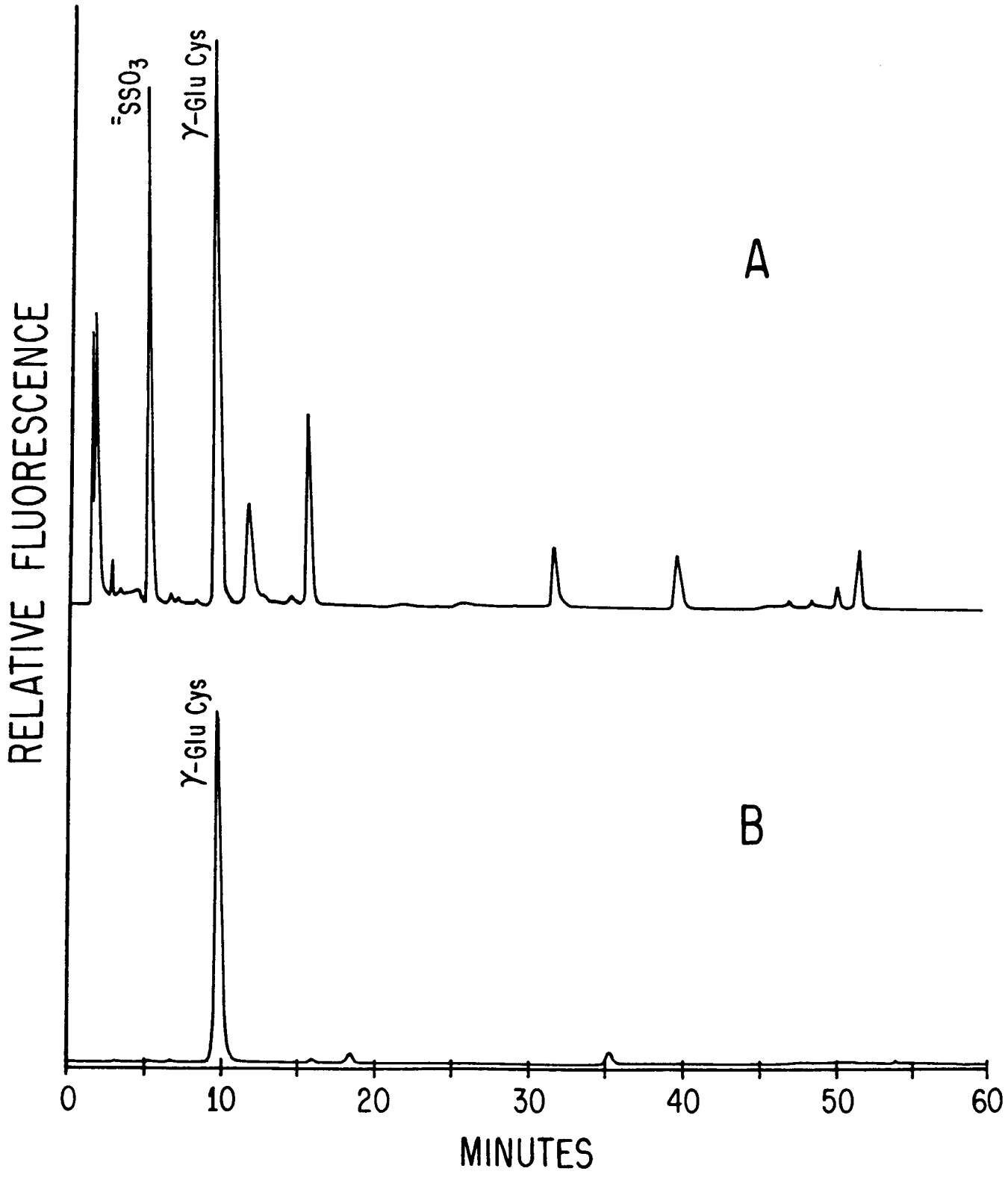
REFERENCES

1. R. C. Fahey and G. L. Newton, in "Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects" (A. Larsson, et al, Eds.), p. 251, Raven Press, New York, 1983.
2. G. L. Newton and B. Javor, J. Bacteriol. 161, 438 (1985).
3. R. Axén, H. Drevin, and J. Carlsson, Acta Chem. Scand. 29, 471 (1975).
4. G. L. Ellman, Arch. Biochem. Biophys. 82, 70 (1959).
5. D. R. Grassetti and J. F. Murray, Arch. Biochem. Biophys. 119, 41 (1967).
6. H. Kadin and R. B. Poet, J. Pharmaceut. Sciences 72, 1134 (1982).
7. A. Meliss, N. S. Kosower, N. A. Crawford, E. Kirowa-Eisner, M. Schwarz, and E. M. Kosower, submitted.
8. N. S. Kosower, G. L. Newton, E. M. Kosower, and H. M. Ranney, Biochim. Biophys. Acta 622, 201 (1980).

CAPTIONS TO FIGURES

Fig. 1. HPLC analysis (method 1, [XX] this volume) of mBBr labeled samples: (A) crude acid-acetonitrile extract of H. halobium R-1 (B) purified mBBr derivative from H. halobium R-1.

Fig. 2. Progress curve for electrolytic reduction of 1 ml 0.2 mM GSH-mBBr derivative at 5 volts: (●) GSH-mBBr derivative assayed by HPLC; (■) GSH assayed by dederivatization with mBBr and HPLC; (▲) syn-(methyl,methyl)bimane assayed by HPLC.


RELATIVE FLUORESCENCE
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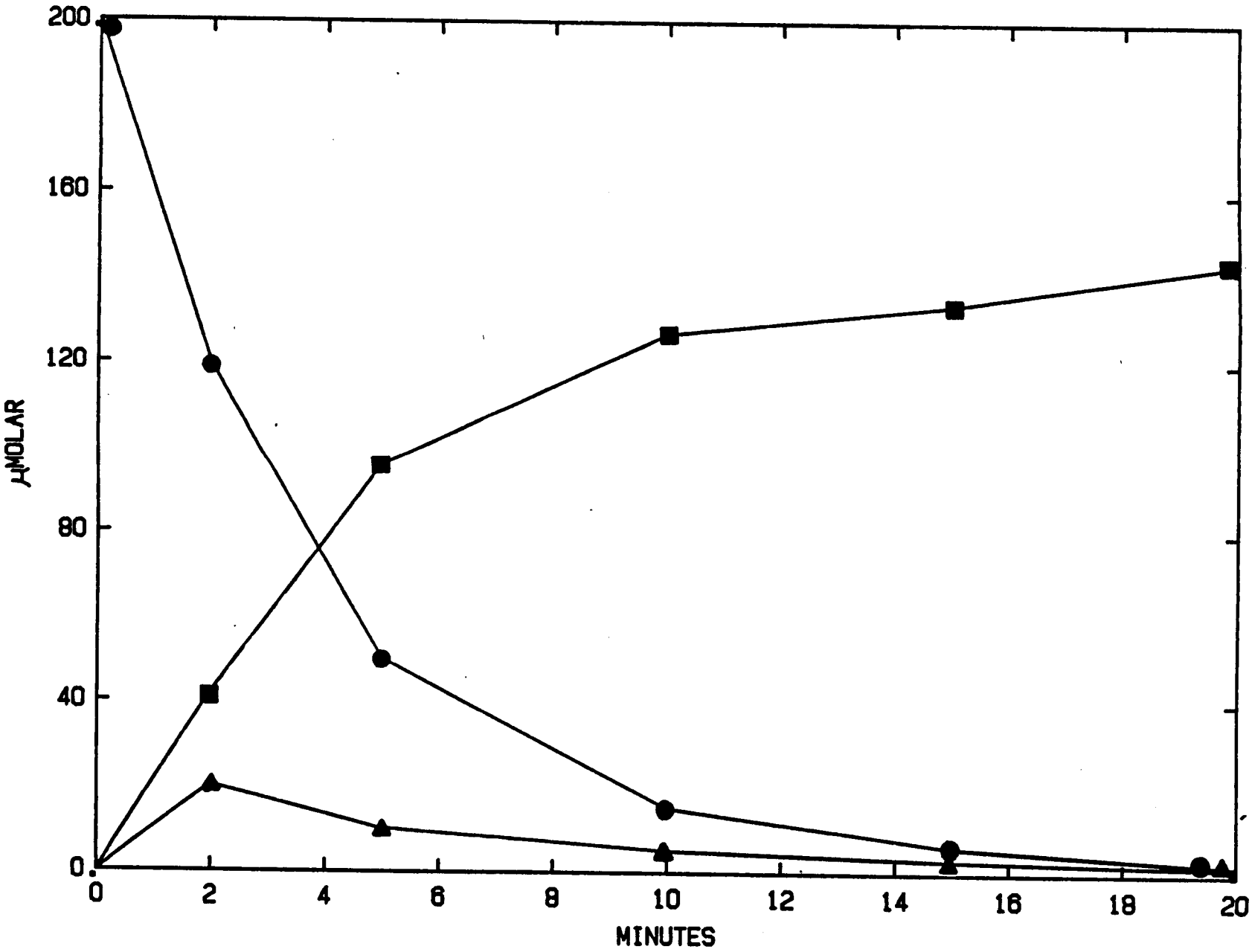
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